(12) UK Patent Application (19) GB (11) 2 247 238 (13) A

(43) Date of A publication 26.02.1992

(21) Application No 9018268.4

(22) Date of filing 20.08.1990

(71) Applicant
Arthur Guinness Son & Company (Dublin) Limited

(Incorporated in Ireland)

St James's Gate, Dublin 8, Ireland

(72) Inventors
Barbara Ann Cantwell
Bridget Therese Kinsella
Angela Larkin

(74) Agent and/or Address for Service
Urquhart-Dykes & Lord
91 Wimpole Street, London, W1M 8AH,
United Kingdom

(51) INT CL[5]
C12N 1/19 // C12N 9/24 15/11 15/56 (C12N 1/19 C12R 1:865)

(52) UK CL (Edition K)
C3H HB1 HB7E HB7T H642 H650 H654 H655 H732
C6Y Y313 Y330
U1S S1111 S1629

(56) Documents cited
ANTONIE VAN LEEUWENHOEK, VOL 51, NO. 3, 1985, PAGES 275-287

(58) Field of search
UK CL (Edition K) **C3H HB1 HB7E HB7T**
INT CL[5] **C12N**
Online databases: DIALOG-WPI, BIOTECH

(54) **Alpha-glucosidase gene from *Candida tsukubaensis* cloned and expressed in *Saccharomyces cerevisiae***

(57) A gene (Figure 5) encoding an amylolytic enzyme (alpha-glucosidase) from *Candida tsukubaensis* has been cloned, sequenced, and expressed in *Saccharomyces cerevisiae*. The cloned material is present on the plasmid pGA8701 (which has been deposited as *S. cerevisiae* AH22(pGA8701) in the National Collection of Yeast Cultures with the number NCYC 2345). The enzyme is heat labile and *S. cerevisiae* strains expressing the enzyme are suitable for use in the production of low carbohydrate beers. The cloned DNA also contains a strong promoter useful for directing the expression of other heterologous genes in *S. cerevisiae*.

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AMYLOLYTIC ENZYME

This invention concerns amylolytic enzymes. More specifically, it concerns the cloning of an α-glucosidase gene from Candida tsukubaensis in Saccharomyces cerevisiae, thereby providing the latter with improved characteristics, making possible the brewing of beer having a reduced carbohydrate content.

There is considerable interest in the brewing industry in the production of superattenuated low calorie beers (so-called "light" beers). Low levels of carbohydrate are achieved in these beers by the addition of fungal amyloglucosidases, generally from Aspergillus niger, Aspergillus awamori, or Rhizopus delemar, either at mashing or during fermentation of the wort. These enzymes convert the non-fermentable dextrins in the wort into glucose, which can be metabolised by the yeast to produce alcohol. Commercial preparations of amyloglucosidase are impure, however, and frequently behave in unexpected ways with unpredictable effects on beer quality. For example, amyloglucosidases may interact with polypropylene glycol alginate in beer to form hazes. Contaminating proteases present in the enzyme preparation may also affect head-retention. Furthermore, most fungal amyloglucosidases are not inactivated by pasteurisation, and may continue to break down dextrins during storage forming sugars which can no longer be fermented, giving rise to sweetness in the beer. Another disadvantage of using commercial enzymes is that they add to production costs. Consequently, there is a requirement for new brewing yeast strains capable of degrading wort dextrins during fermentation.

Both rare mating and protoplast fusion techniques have been used to generate hybrids between brewing yeast and the amylolytic yeast, Saccharomyces diastaticus. Brewing strains with similar fermentation and organoleptic characteristics to the parental yeast, plus exogenous amyloglucosidase, can be produced by these methods only if the POF1 gene, which is responsible for a detrimental "phenolic" off-flavour in beer, is eliminated . Recombinant DNA techniques offer a more defined, specific route to the genetic modification of brewer's yeast. Perry and Meaden (J. Inst. Brew. 94, 64-67) have cloned the DEX1 (or STA2) gene of S.diastaticus into both lager and ale yeast and fermentation trials show that the yeast transformants can degrade up to 30% of the wort dextrins. This corresponds to the degree of hydrolysis which one might expect from an enzyme which does not exhibit any α-1, 6 activity, and hence cannot completely break down starch and related oligosaccharides.

Genes coding for amyloglucosidases with debranching activity have been isolated from other yeasts and filamentous fungi, including Saccharomycopsis fibuligera, Candida albicans. Aspergillus awamori, Aspergillus niger and Rhizopus sp. Expression and secretion of active amyloglucosidase has been achieved from laboratory strains of Saccharomyces cerevisiae transformed with the cloned genes. With the exception of the amyloglucosidase genes from S.diastaticus and A.niger, none of these genes have yet been introduced into brewing strains of yeast. Unfortunately, all of these enzymes suffer from the disadvantage that they are not completely destroyed by pasteurisation.

Various genes from Candida species have, in the past been transferred into Saccharomyces cerevisiae. Kawamura et al. in Gene. 24(1983) 157-162 describe the cloning of a LEU gene from C. maltosa. Hsu et al. in Journal of Bacteriology 154(1983), 1033-1039 describe the cloning of several DNA fragments from C. utilis into YIp5, thereby conferring onto YIp5 the ability to replicate autonomously in S. cerevisiae. Loper et al. in a paper published in US Environ. Prot. Agency, Res. Dev. (Rep)EPA (EPA/600/9-84/015, Incineration treat, Hazard Waste), pp 274-281, describe the cloning of gene sequences from C. tropicalis into S. cerevisiae. Gillum et al. in Mol. Gen. Genet. (1984) 198: 179-182 describe isolation of a gene for orotidine - 5- phosphate decarboxylase by complementation of S.cerevisiae ura 3 mutations. EP-A-0173668 relates to the expression of certain functional DNA sequences from Candida species into Saccharomyces cerevisiae, especially as a means of studying agents for the treatment of Candidiasis infections.

De Mot et al (Antonie van Leeuwenhoek, 51, 275-287) have shown that the filamentous yeast Candida tsukubaensis CBS 6389 produces an extracellular heat labile amyloglucosidase.

We have now isolated a gene coding for a novel amylolytic enzyme from a genomic library of Candida tsukubaensis. We have expressed the gene in Saccharomyces cerevisiae. This enzyme has properties which make it suitable for use in the production of

low carbohydrate beers. It is different in several respects from the amyloglucosidase enzyme mentioned by de Mot, as will be demonstrated below.

One embodiment of the present invention provides Saccharomyces cerevisiae having an α-glucosidase gene from Candida tsukubaensis CBS 6389.

A further embodiment of the invention provides a method of providing Saccharomyces cerevisiae with enhanced α-glucosidolytic activity comprising transforming said Saccharomyces cerevisiae with an α-glucosidase gene from a genomic library prepared from Candida tsukubaensis. Another embodiment of the invention provides a method of fermenting carbohydrate material comprising adding a culture of S.cerevisiae to an aqueous solution, suspension or dispersion of said carbohydrate material wherein the S.cerevisiae has an α-glucosidase gene derived from Candida tsukubaensis.

Another embodiment of the invention provides a 4.39 Kb Bam Hl fragment having the nucleotide sequence set out in the accompanying Drawings.

Yet another embodiment of the invention provides a Bam Hl-Xhol fragment extending from nucleotide 1-272 of the above-identified 4.39 Kb BamHl fragment.

A further embodiment of the invention provides α-glucosidase obtained in an extracellular fraction of a culture of S. cerevisiae modified by a gene for C.tsukubaensis CBS 6389, as defined above.

The attached Drawings are provided to illustrate the invention, and will be explained more fully below.

Figs 1(a) and 1(b) are growth curves of clones showing amylolytic enzyme activity.

Fig. 2 is a restriction map of a 4.39 Kb Bam Hl fragment from a transformed yeast according to the invention;

Fig. 3 is a further restriction map;

Fig. 4 is a graphical illustration of a strategy for sequencing the 4.39 Kb fragment;

Fig. 5 is the nucleotide sequence of the 4.39 Kb fragment.

Fig. 6 shows amino acid sequences derived from the peptide coded by the 4.39 Kb fragment.

Candida tsukubaensis CBS 6389 was first described by Onishi in Antonie van Leeuwenhoek, 38 (1972), 365-367.

Chromosomal DNA was prepared from C. tsukubaensis by physical disruption of the yeast cells. Chilled cells were ground and solvent-extracted. Nucleic acids from the extract were digested with RNase in conventional manner.

The chromosomal DNA was partially digested with restriction endonuclease Sau 3A, and fractionated. Fragments from 2.5 to 20 Kilobases were used to compile the genomic library by ligation to Bam HI digested, dephosphorylated YEp 13 with T4 DNA ligase. The resulting hybrid DNA was used to transform Escherichia coli.

The following abbreviations are used herein:

Ci, Curie; cpm, counts per minute; ^{o}C, degree Centigrade; [α-^{32}P] dGTP, Deoxyguanosine 5'-[α-^{32}P] triphosphate; DNA, Deoxyribonucleic Acid; EDTA, Ethylenediamine tetra acetic acid; H, Histidine; Kb, Kilobase pair; L, Leucine; OD, Optical density; PAGE, Polyacrylamide gel electrophoresis; rpm, revolutions per minute; SDS, sodium dodecyl sulphate; Tris, Tris (hydroxymethyl) methylamine; T, Tryptophane; U.V., Ultra violet.

SSC refers to a solution containing sodium chloride and sodium citrate. The method of preparation is given in Maniatis et al. (1982): Molecular cloning : a laboratory manual, pub. Cold Spring Harbor Laboratory, NY.

In the following, yeast strains were grown at 30°C in complete medium (YEPD) containing 20g glucose per litre or in minimal medium containing Difco yeast nitrogen base w/o amino acids (0.67%) and either 2% glucose (YNBG) or 2% maltose (YNBM) as sugar source. Histidine (20 μg ml^{-1}), leucine (30 μg ml^{-1}) uracil (20 μg ml^{-1}) and tryptophane (20 μg ml^{-1}) were added as required.

EXAMPLE 1

Preparation of chromosomal DNA

Because C.tsukubaensis cells were resistant to spheroplast formation using enzymatic treatment of the yeast cell wall, chromosomal DNA was obtained by a method involving physical disruption of the yeast cells. All centrifugation steps were carried out in a Sorvall SS-34 rotor using a Sorvall RC-5B

refrigerated superspeed centrifuge at 4°C. C.tsukubaensis cultures (100 ml) which had been grown to stationary phase in YNBD at 24°C for 6 days were harvested by centrifuging at 10,000 rpm for 10 minutes and washed once by resuspension in cold, distilled H_2O. The cell pellet was drained of excess liquid and ground to a fine powder using a mortar and pestle and at least 1 litre of liquid nitrogen. The powder was then transferred carefully to a sterile centrifuge bottle and resuspended gently in 100mM Tris-HCl, pH 7.4., 1mM EDTA., 1% (w/v) SDS at a final ratio of 5g wet weight of cells/100 ml solution. The viscous suspension was extracted three times with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1 v:v:v: equilibrated in 10mM Tris-HCl, pH 8.0, 1mM EDTA), with intermediate separation of the aqueous and organic phases by centrifuging at 12,000 rpm for 10 minutes and finally, extracted once with an equal volume of chloroform:isoamyl alcohol, (24:1 v:v). Nucleic acids were precipitated by adjusting the aqueous fraction to 0.1M NaCl (from a 5 M NaCl stock solution) followed by the addition of 2 volumes of cold absolute ethanol (-20°C), and were subsequently collected by centrifuging at 7,000 rpm for 10 minutes. The resulting nucleic acid pellet was dried in vacuo and redissolved in 10mM Tris-HCl, pH 8.0, 1mM EDTA (TE, pH8.0) Pancreatic RNase A (Sigma, 10 mg/ml in 10mM Tris-HCl, pH 7.5, 15mM NaCl heated to 100°C for 15 minutes to render DNase free and stored in aliquots at -20°C) and T1 RNase (Sigma, 200, 000 U/ml in 2.7 M $(NH_4)_2SO_4$, 20mM Tris-HCl, pH 6.0) were added to final concentrations of 100 ug/ml and 100 U/ml, respectively, and RNA was digested during an overnight incubation at 4°C. The nucleic acid mixture was then further deproteinized by extraction (twice) with equal volumes of phenol:chloroform with separation of the phases by centrifuging at 7,000 rpm for 10 minutes followed by a chloroform extraction. High molecular weight chromosomal DNA was precipitated by

adjusting the aqueous fraction to 0.1M NaCl (from a 5M NaCl stock) followed by the addition of 0.6 volumes of isopropanol. After 15 minutes at room temperature, the DNA was collected by centrifuging at 7,000 rpm for 10 minutes. The chromosomal DNA pellet was rinsed once in 70% (v/v) aqueous ethanol, dried in vacuo and redissolved in a sterile TE buffer (pH 8.0).

Plasmid DNA isolation and transformations

Plasmid DNA was isolated from E.coli using the rapid alkaline lysis extraction procedure of Birnboim and Doly Nucl. Acids Res., 7(6) (1979) 1513-1523, and purified by CsCl-ethidium bromide equilibrium density gradient centrifuging. E.coli was routinely transformed by the $CaCl_2$ competent cell method of Mandel and Higa as described by Lederberg and Cohen: J. Bacteriol; 119(3) (1974) 1072-1074. Yeast spheroplasts were transformed essentially as described by Hinnen et al. Proc. Natl. Acad. Sci. USA 75(4), (1978) 1929-1933, while intact yeast cells were made competent and transformed using the lithium acetate method of Ito et al: J. Bacteriol; 169(9) (1983) 4171-4176. Plasmid DNA was recovered from transformed yeast cells as described by Hoffman and Winston: Gene, 57 (1987) 267-272 and used to transform E.coli using the $CaCl_2$ /RbCl procedure of Kushner as set out in "Genetic Engineering" Ed. Boyer & Nicosia, Elsevier (1978) pp. 17-23.

Construction of C.tsukubaensis genomic library

Candida tsukubaensis chromosomal DNA (100 µg) was partially digested with the restriction endonuclease Sau 3A, using a ratio of enzyme:DNA of 0.1:1 units/µg DNA at 37°C for 10 minutes, and was subsequently fractionated on a linear 5-20% (w/v) sucrose gradient (in 10ml of 20 mM Tris-HCl. pH 8.0,, 5mM EDTA, 1M NaCl)

by equilibrium density gradient centrifuging (39,000 rpm for 5.5 hours at 15-19°C in a Sorvall TH 641 rotor). Individual gradient fractions (0.5ml) were collected, aliquots were analysed by agarose gel electrophoresis and fractions containing DNA fragments ranging from 2.5-20 kilobases (Kb) were pooled and used for library construction. These DNA fragments were ligated to BamHI digested, dephosphorylated YEp13 with T4 DNA ligase at an insert to vector ratio of 1:1 (12.5 ug:12.5 ug insert:vector). This pool of hybrid DNA molecules was used to transform E.coli JA221 to AMP^r and the transformants were stored as pools at -70°C in 15% glycerol.

DNA hybridisation

DNA depurination, denaturation, and neutralization followed by Southern transfer of fractionated chromosomal DNA digestions from agarose gels onto nylon filters (Hybond N; Amersham) were carried out as described by Southern in J.Mol. Biol. 98 (1975), 503-517 but included modifications recommended by the supplier when using nylon membranes. A radiolabelled DNA probe was prepared by the incorporation of α-^{32}P-dGTP (10mCi / ml, 3,000 Ci/ mmol) into the recessed 3' ends of BamHI digested plasmid pGA8701 in a fill - in reaction catalysed by the Klenow fragment of E.coli DNA polymerase: Drouin: J. Mol. Biol, 140(1980), 15-34. The 4.3 kb BamHI radiolabelled probe (subfragment of the 6.2 kb Sau3A cloned fragment) was separated from host vector sequences by fractionation on 0.8% agarose followed by DNA extraction of the required fragment from the gel, according to Vogelstein and Gillespie, Proc. Natl. Acad. Sci. USA, 76(2), (1979), 615-619. Filters were prehybridised for 3-4 hours at 65°C in 6 X SSC, 5 x Denhardt's solution, 0.5% (w/v) SDS containing the denatured radiolabelled probe (heated to 100°C for 15 minutes and cooled; 10^6-10^7 cpm/ml hybridisation solution). Filters were

subsequently washed under conditions of successively increasing stringency, by raising the washing temperature and/or decreasing the salt concentrations in the washing solution, followed by autoradiography at -70°C with Fuji RX film and intensifying screens.

Enzyme Assays

Amylolytic enzyme activity measurements were based on the colorimetric determination of free glucose liberated from 2% (w/v) substrate in 50mM of McIlvaine buffer, at the specified pH, time and temperature. Standard assay conditions were 60 minutes at 50°C using 2% maltose or 2% dextrin (in 50mM of McIlvaine buffer, pH 5.4) as substrate. The reaction was stopped in boiling water for 10 minutes and the glucose concentration estimated using the Glucose oxidase - Peroxidase diagnostic kit (Sigma Diagnostics Procedure N°510) or that supplied by Boehringer Mannheim GmbH (Test-combination glucose). One unit of enzyme activity is defined as that amount of enzyme which liberates 1 uMol of D-glucose per hour under the conditions of the assay.

Molecular cloning of a gene coding for an amylolytic enzyme from C.tsukubaensis

The genomic library constructed in E.coli JA221 contained some 40-50,000 independent transformants, 94% of which were judged to be recombinants due to insertional inactivation of the vector based tetracycline gene. As the Candida tsukubaensis genome is about 2×10^4 kb in size, 8×10^3 independent transformants carrying an insert of about 10 kb are required to provide a genomic library in which each of the C.tsukubaensis gene sequences will be represented at least once with a 99%

probability. On the basis of this estimation, the C.tsukubaensis - YEp13 library should contain more than one copy of every gene. Recombinant library (Candida tsukubaensis-YEp13) plasmid DNA was amplified and purified from E.coli JA221 transformants, and aliquots of the library DNA were used to transform S.cerevisiae AH22 with initial selection of yeast transformants based on complementation of the leu 2 gene. Three individual libraries of S.cerevisiae AH22 (C.t - YEp13) transformants were generated, and each library was estimated to contain 1.0 to 1.5 x 10^4 LEU^+ transformants. S.cerevisiae AH22 LEU^+ transformants were recovered from the regeneration media, were pooled and inoculated directly into YNBG,H medium. The transformants were then grown overnight at 30°C.

Fractions of the S.cerevisiae AH22 (C.t. - YEp13) library cultures were then either stored at -70°C in 15% glycerol or plated directly onto appropriate selection media for the identification of recombinants expressing the desired phenotype. Detection of S.cerevisiae AH22 transformants with amylolytic properties was initially based on screening for colonies growing on synthetic media containing either dextrin (YNBD,H) or starch (YNBS,H) as the major carbon source. Fractions of the pooled S.cerevisiae AH22 (C.t. - YEp13) recombinant libraries, represented by some 3 to 4.5x10^4 independent transformants, and serial dilutions thereof were plated on the appropriate selection medium (YNBD, H or YNBS,H) at cell densities ranging from 2x10^3 to 2x10^5 cells/plate, and the plates were incubated at 30°C. After 10 days incubation at 30°C, two very strongly growing colonies were evident on dextrin - containing plates (these clones were named DEX 1 and DEX 2). Each of these putative 'amylolytic clones' was confirmed to have a HIS^- phenotype and was morphologically identical to the host strain S.cerevisiae AH22 on microscopic examination. Initial characterisation of

amylolytic clones was based on comparisons of their growth rates on selection plates containing either dextrin or starch as sole carbon sources relative to the growth displayed by the non-amylolytic S.cerevisiae AH22 host strain (Meaden et al., Gene, 34(1985), 325-334), which acted as a negative control, and that of the amylolytic yeast S.diastaticus ATCC 28339 which acted as a suitable positive control. DEX 1 and DEX 2 grew better on both dextrin and starch media than the control strain, S.cerevisiae AH22; and DEX 1 and DEX 2 appeared to grow marginally faster on dextrin than on starch. In addition, they produced clear haloes around areas of growth on starch plates held at 4°C for 2-3 days, similar to the clearing displayed by the control strain S.diastaticus ATCC 28339, indicating starch degradation. On the basis of this, clones DEX 1, and DEX 2 were studied for dextrinase activity and cell density. Exponentially growing cultures were inoculated into a selective synthetic medium containing dextrin (YNBD,H) and incubated at 30°C. Cell densities (Figure 1(a)) of the recombinant cultures S.c. AH22 DEX 1 (■), S.c. AH22 DEX 2 (+)., and S.c. AH22 YEp13 (*) as control were estimated by measuring the absorbance at 600nm ($OD_{600\ nm}$/ml). Dextrinase activity (Figure 1(b)) in the corresponding culture supernatants was measured by determining the glucose liberated (μmoles) per ml enzyme solution from 2% dextrin (in 50 mM McIlvaine buffer, pH 5.4) at 50°C in 1 hour.

The favourable growth rates of clones DEX 1 and DEX 2 relative to that of S.cerevisiae AH22 (YEp13) in this medium coupled to the detection of low levels of dextrinase activity (1-2 μmoles glucose / ml liberated in 1 hour at 50°C) in the culture supernatants of DEX 1 and DEX 2 was taken as positive confirmation that these clones contain a gene encoding an enzyme capable of degrading dextrin and that this gene is expressed and secreted by S.cerevisiae.

Plasmid curing

Plasmid curing experiments were carried out to confirm that the amylolytic properties of DEX 1 and DEX 2 were due to a recombinant plasmid gene function and not to a mutation in the genome of the host strain S.cerevisae AH22. Each clone was initially grown for about 10 generations (15-16 hours) non-selectively at 30°C in MYGP medium, plated onto complete synthetic medium (YNBG, H, L) and incubated at 30°C for a further 3 days. Individual colonies were tested for the concomitant loss of both the leu 2 gene (by plating onto YNBG medium lacking leucine) and of the ability to grow on dextrin as sole carbon source (by plating onto YNBD supplemented with both leucine and histidine). Of a total of 100 DEX 1 colonies tested, 49 colonies had lost the LEU 2 phenotype and the ability to grow on dextrin. The remaining colonies retained the ability to complement leu 2 and the ability to grow on dextrin. Similarly, of a total of 100 DEX 2 colonies tested, 53 colonies which retained the plasmid LEU 2 phenotype also retained the dextrinase phenotype. DEX 2 colonies which had been cured of the plasmid failed to grow on dextrin medium.

Further confirmation of these results was provided by examination of a random sample of 40 colonies originating from DEX 1 and DEX 2 cultures, 20 of which were found to have retained and 20 of which had lost the plasmid LEU2 phenotype. The individual colonies were grown in synthetic medium containing dextrin (YNBD,H) at 24°C for 7 days and then monitored for growth by measuring the increase in optical density at 600nm and production of dextrinase activity in culture supernatants.

In all cases examined, colonies which retained the LEU+ phenotype were found to grow better on YNBD medium, reaching cell densities (assessed via the increase in optical density) some 3 to 4 fold greater than colonies which had lost the plasmid. In addition, the former colonies secreted low but detectable levels of dextrinase into the culture medium. These results confirmed that the ability to degrade dextrin in the medium was due to the presence of a recombinant plasmid in DEX 1 and DEX 2 clones.

Transformation of S.cerevisiae DBY746

To obtain further confirmation that the ability of DEX 1 and DEX 2 to degrade dextrin in the medium was due to a plasmid encoded function, it was necessary to establish if plasmids recovered from these clones could confer similar amylolytic properties on other strains of S.cerevisiae. DNA recovered from freshly grown cultures of DEX 1 and DEX 2 (grown in YNBG,H, at 30°C for 24 hours) was initially transformed into E.coli JA221 in order to facilitate plasmid amplification and purification. Plasmid DNA which originated from DEX 1 was designated pGA8701 and that from DEX 2 was designated pGA8702. Plasmid DNA isolated from the AMP^r, TET^s E.coli transformants was then used to transform competent cells of S.cerevisiae DBY746 with selection for complementation of the LEU^- phenotype. Individual LEU^+ transformants were then analysed for their ability to grow in synthetic medium containing dextrin (YNBD, H,U,T) at 30°C; cell densities (OD_{600nm}) were measured and the culture supernatants were assayed for the presence of dextrinase activity. All of the S.cerevisiae DBY 746 transformants containing pGA8701 or pGA8702

achieved cell densities some 2 to 3 times greater than the control strain S.cerevisiae DBY746 (YEp13) following 3 days incubation at 30°C in selective YNBD medium; the low, but detectable levels of dextrinase activity present in the culture supernatants of the former strains was interpreted as confirmation that the production of amylolytic enzyme by DEX 1 and DEX 2 was due to C.tsukubaensis DNA sequences cloned in YEp13.

Restriction endonuclease mapping

A restriction endonuclease map of the plasmids pGA8701 and pGA8702 originating from DEX 1 and DEX 2 was prepared in order to characterize the recombinant DNA inserts. Initial restriction endonuclease mapping experiments revealed that the recombinant plasmids pGA8701 and pGA8702 each contained a 6.2 kb DNA insert flanked by Sau 3A recognition sites. More detailed analysis of the recombinant DNA showed that the plasmids shared identical restriction patterns for BamHI, SalI and HindIII and that both recombinant plasmids shared the same insert orientation with respect to host vector sequences. In view of the near identical behaviour of the recombinant clones DEX 1 and DEX 2 with respect both to growth characteristics on YNBD and to production of amylolytic enzymes, it was assumed that DEX 1 and DEX 2 were independent isolates of the same clone. Consequently only plasmid pGA8701, isolated from DEX 1, was subjected to further analysis. Single and double restriction digests of pGA8701 with various restriction endonucleases showed that the 6.2kb cloned insert contained 4 sites for SalI, 3 sites for PvuI, 2 sites for BamHI, EcoRI and HindIII and 1 site for XhoI and PstI. There were no sites on the cloned fragment for HpaI, PvuII, XbaI or BclI. Fig. 2 is detailed restriction map of the insert in pGA8701.

In addition to the recognition sites given in Fig. 2, there are no sites on the cloned fragment for the restriction endonucleases Hpa I, Pvu II, Xba I or Bcl I. The Sau 3A restriction endonuclease sites, at nucleotide positions 1, 6200 bp, which flank the cloned insert present in the recombinant plasmid pGA8701 are included but internal Sau 3A recognition sites were not mapped.

Southern blot analysis

In order to verify the genetic origin of the cloned fragment encoded by the recombinant plasmid pGA8701 and to investigate the existence of possible homologies between the insert fragment and DNA sequences from other strains of yeast, a radiolabelled subfragment of the 6.2 kb cloned insert was used to probe total genomic DNA isolated from a variety of yeast strains. Chromosomal DNA was prepared from S.diastaticus ATCC 68339, S.cerevisiae AH22, S. cerevisiae DBY746, S.cerevisiae 1164 (ale yeast), S.cerevisiae 3804A (lager yeast), Sch.occidentalis CBS 819 and C.tsukubaensis CBS 6389. Aliquots (10 µg) of each preparation were digested to completion with the restriction endonuclease BamHl and fractionated by agarose gel electrophoresis. The DNA fragments were transferred to a nylon filter and probed with a ^{32}P-labelled 4.3 kb BamHI fragment isolated from pGA8701.

The hybridisation results clearly showed that the labelled probe specifically hybridised to a 4.3 kb DNA fragment from the BamHI digested chromosmal DNA from C.tsukubaensis CBS 6389, even under highly stringent washing conditions (by washing the filters successively in 2 X SSC 15 minutes; 2 X SSC, 0.1% (w/v) SDS, 30 minutes; 0.1 X SSC, 10 minutes at a

hybridisation temperature of 65°C) thereby providing confirmation of the genetic origin of the cloned fragment in pGA8701.

No hybridisation was observed between the labelled probe and the other specimens of yeast chromosomal DNA screened, even under low stringency washing conditions (by washing the filters successively in 6 X SSC 5 minutes; 6 X SSC 0.1% (w/v) SDS, 2 x 5 minutes; 6 x SSC, at 37°C) indicating the complete absence of homology between the cloned gene fragment and other yeast DNA, including the amylolytic yeast strains S.diastaticus ATCC 28339 and Sch.occidentalis CBS 819.

Characterisation of properties of the cloned enzyme

The biochemical properties of the enzyme encoded by pGA8701 from DEX 1, were examined and compared with values reported for the amyloglucosidase secreted by the donor strain C.tsukubaensis referred to above and described by DeMot et al. Antonie van Leeuwenhoek 51(1985), 275-287.

Determination of substrate specificity

Culture supernatant concentrates of S.cerevisiae AH22 (pGA 8701) grown in YNBD, H were used as enzyme source. Fractions were assayed for enzyme activity using 2% (w/v) substrates (in 50mM McIlvaine buffer, pH 5.4) at 50°C for 30 minutes; activities were calculated on the basis of the amount of D-glucose (µmoles) liberated per ml enzyme solution in 30 minutes. Relative activities were expressed as percentages (%) relative to the enzymic activity displayed with 2% (w/v) maltose as substrate under the specified conditions.

Table 2 sets out the relative activities of the cloned enzyme towards 22 different substrates. Four classes of enzymatic activity for the cloned enzyme may be distinguished on the basis of the type of sugar linkage hydrolysed. The enzyme displayed an unusually broad substrate specificity, being capable of acting on α(1,4), α(1,6), α(1,3), and α(1,2) linkages, as well as on aryl - and to a lesser extent on alkyl-α-D-glucosides. A marked preference for the lower molecular weight oligomeric substrates was evident, which quite clearly distinguishes this enzyme from the amyloglucosidase of C.tsukubaensis CBS 6389 described by DeMot et al., and also from other amyloglucosidases which typically exhibit substrate preferences for the more complex carbohydrates of dextrins and starches. Maltose was the preferred substrate, with comparatively high activities displayed towards the tri - and tetra - glucose oligomers of maltotriose (59%) and maltotetraose (48%), respectively. Dextrin acted as a moderately poor substrate (activities ranging from 6.4 to 8.0% relative to maltose, depending on the preparation of dextrin examined) and the enzyme displayed little or no affinity for starch (0 to 0.7% activity relative to maltose depending, on the starch preparation used); pullulan was not hydrolysed. The amyloglucosidase produced by C.tsukubaensis CBS 6389 described by de Mot displayed an entirely different substrate specificity profile, with dextrin and starch featuring as the preferred substrates. In fact, the substrate specificity profile of our cloned enzyme appears to resemble the profiles exhibited by α-glucosidases more closely than those of amyloglucosidases. Alpha-glucosidases (α-D-glucoside glucohydrolase E.C. 3.2.1.20), have been defined as enzymes which hydrolyse terminal, non reducing α-1,4 and α-1,6 linked glucose

residues in different substrates releasing α-D-glucose; they degrade disaccharides and oligosaccharides rapidly, relative to larger structures and polysaccharides are attacked slowly if at all. Characteristically, although α-D-glucosidases are believed to be highly specific for glucose residues, their specificity for the aglycon portion of the substrate is not as distinct and thus, maltose, maltose derivatives, sucrose and related fructosyl-α-glycosides, aryl - and alkyl-α-glucosides may act as substrates to varying degrees depending on the particular enzyme isolate. On the basis of the above definition, our cloned enzyme may be classified as an-α-glucosidase, in that maltose and derivatives are the preferred substrates, with the more complex polysaccharides being hydrolysed more slowly or not at all (Table 2). Sucrose is a moderately good substrate (55% activity relative to maltose) with the derivative melezitose hydrolysed somewhat more slowly (8.8% activity relative to maltose). Our cloned enzyme hydrolysed phenyl-α-D-glucoside and α-methyl-D-glucoside at rates of 18% and 0.7% relative to that of maltose, respectively. It also displayed relatively good hydrolysis of α-(1,6) linked glucose units as indicated by the relative activities of 35% with isomaltose (α-D-glucose (1,6)-α-D-glucose) and that of 55% with panose (α-D-glucose (1,6)-α-D-glucose - (1,4)-D-glucose) and, interestingly, also displayed some activity towards (1,3) linkages, as indicated by the hydrolysis of turanose (4.1% activity relative to maltose).

Determination of pH optimum

The activity of the enzyme at pH 3.4, 3.9, 4.2, 4.4, 4.6, 4.9 and 5.4 was determined. The optimum was found to be within the range from 4.2 to 4.6, (see Table 3). No sharp

dependence on pH was observed, with more than 90% of enzyme activity remaining over a range of 1 pH unit from pH 3.9 to 4.9. The enzyme clearly differs from the amyloglucosidase of C.tsukubaensis CBS 6389, which displayed highest activity over a much broader pH range of 2.4 to 4.8.

Determination of temperature optimum

The activity of the enzyme was examined over a range of temperatures (20 to 65°C) in order to determine its optimum temperature. The results obtained (Table 4) clearly indicated that the optimum temperature lay within the range from 55 to 60°C, with marginally greater activity displayed at 58°C. Increasing the temperature of incubation to 65°C resulted in a sharp inactivation of the enzyme, leaving only 40.4% of the activity observed at 58°C. De Mot et al. reported that the purified amyloglucosidase secreted by the donor strain C.tsukubaensis CBS 6389 had a temperature optimum of 55°C with relative activities of 70% and 26% observed at 60°C and 65°C respectively.

Thermostability at 60°C

For the determination of the thermostability of the cloned α-glucosidase enzyme, S.cerevisiae AH22 (pGA 8701) was grown in dextrin containing medium (YNBD, H) for 3 days at 24°C; culture supernatants were assayed at 55°C for 1 hour using 2% maltose (in 50 mM McIlvaine buffer, pH 5.4) as substrate following preincubation at 60°C for 0,1,2,5,10,20, and 60 minutes. The results obtained clearly indicate (Table 5) that the enzyme, when grown in YNBD,H, containing medium, was inactivated following incubation at 60°C for 5 minutes. Similar results were reported by De Mot et al. for the amyloglucosidase from C.tsukubaensis CBS 6389.

Estimation of molecular weight

The protein profiles of culture supernatants of S.cerevisiae AH22 (pGA 8701) were analysed and compared with the corresponding protein profiles from culture supernatants of a control strain S.cerevisiae AH22 (YEp13) by analytical SDS polyacrylamide gel electrophoresis in order to identify the cloned α-glucosidase gene product. The levels of α-glucosidase activity produced by S.cerevisiae AH22 (pG8701) grown in either dextrin, maltose or glucose containing synthetic medium were equivalent and those levels of α-glucosidase enzyme activity expressed were directly proportional to the cell densities of the cultures when grown under identical conditions of temperature and time, thereby suggesting that expression of the cloned α-glucosidase gene is constitutive and is not subject to glucose repression. Thus in order to facilitate growth of the control strain to cell densities comparable to those of the recombinant S.cerevisiae AH22 (pGA 8701), cultures (100 ml) were grown in a synthetic medium containing glucose as carbon source (YNBG, H) at 24°C for 3 days. Protein was concentrated 500-fold from the culture supernatants (after centrifuging at 5,000 rpm, 10 minutes, 4°C) by repeated acetone precipitation followed by solution in a minimal volume of double distilled H_2O. The protein contained in 5-50 ml of culture supernatant was then analysed by SDS-PAGE. Direct comparison of the protein profiles revealed the presence of a somewhat diffuse protein band in the culture supernatants of the recombinant strain S.cerevisiae AH22 (pGA 8701) which was not present in the supernatant of the control strain S.cerevisiae AH22 (YEp 13). The molecular size of the proposed α-glucosidase peptide was estimated to be about 75,000

daltons. On treatment of the above culture supernatant concentrates with Endoglycosidase H, two bands were evident in the protein profiles of the recombinant strain S.cerevisiae AH22 (pGA8701) which were not present in that of S.cerevisiae AH22 (YEp 13) control and not present in corresponding untreated controls. The molecular sizes of the two polypeptides were judged to be 65,000 (peptide 1) and 52,000 (peptide 2) daltons, approximately. This suggests that pGA 8701 encodes not one but two protein products which are glycosylated in S.cerevisiae AH22.

Localisation of the α-glucosidase gene on pGA 8703

Plasmids pGA8703 and pGA8704 differing only in the orientation of the insert relative to the vector plasmid were constructed by transferring a 4.39 Kb BamHl fragment of C.tsukubaensis DNA which contains the C.tsukubaensis α-glucosidase gene from pGA8701 into the BamHl site of YEp24 and transformed into S.cerevisiae DBY 746. The restriction map of this 4.39 Kb BamHl insert is shown in Fig. 2 of the accompanying Drawings. To localise the α-glucosidase gene, various deletion derivatives were constructed in YEpl3 and introduced into S.cerevisiae AH22. Plasmids pGA8801/pGA8802 were constructed by first subcloning a Pstl fragment containing the yeast Ura3 gene and a BamHl-Pstl fragment of Candida DNA from pGA8703 into pUC1318 to give pGA8710. The BamHl-Pstl fragment was then recovered from pGA8710 as a BamHl fragment and inserted into YEpl3. The plasmids pGA8812/pGA8813 were derived from the 4.39 Kb BamHl fragment by deleting an internal 1.6 Kb Pvul fragment and transferring the deleted fragment into YEpl3. BamHl linkers were added to the Ndel and Mlul sites flanking the BamHl-Ndel and BamHl-Mlul fragments before subcloning into the BamHl site of YEpl3 to generate the recombinant

plasmids pGA8817/pGA8818 and pGA8912/pGA8913. The S. cerevisiae transformants were screened for ability to grow in yeast minimal medium containing maltose as sole carbon source and for the presence of ∝-glucosidase activity in culture supernatants.

Only recombinant plasmids which contained the entire 4.39 Kb BamHl fragment or the 3.46 Kb BamHl-Mlul fragment produced ∝-glucosidase. Moreover, ∝-glucosidase activity was detected only when the BamHl or BamHl-Mlul fragments were inserted into the cloning vectors YEp24 or YEpl3 in the same relative orientation i.e. the reverse orientation with respect to the direction of transcription initiation from the Tet^r gene promoter. These results, together with other observations on the expression of ∝-glucosidase S.cerevisiae recombinant plasmids containing the yeast GAL 1-10 promoter and CYC-1 terminator sequences, led to the conclusion that the direction of ∝-glucosidase gene transcription was, 5' - 3', from the BamHl site at nucleotide 1 through to the Mlul site at nucleotide 3,468. In addition, because the relative levels of ∝-glucosidase activity expressed by the positive recombinant plasmids were roughly equivalent, irrespective of whether the entire 4.39 Kb BamHl fragment or the smaller BamHl-Mlul fragment was present, it was evident that the 3.46 Kb BamHl-Mlul fragment contained the entire DNA sequence necessary for the expression of the ∝-glucosidase gene in S.cerevisiae.

DNA sequence determination

The nucleotide sequence of the∝-glucosidase gene transferred was determined in conventional manner, and is set out in Fig. 5 of the accompanying Drawing.

In order to facilitate DNA sequence determination of the entire 4.39 Kb BamHl fragment containing the ∝-glucosidase gene, the series of recombinant plasmids pTS 001 to pTS 015 was constructed by cloning subfragments into the appropriate sitess within the polylinker region of the vector pUC8 as illustrated in Fig. 3.

To facilitate construction of plasmids pTS014 and pTS015, BamHl linkers were added to the filled in Ndel sites before inserting the fragments into pUC8. DNA inserts from a selection of these plasmid constructs were then cloned directly into the

bacteriophage M13 derivatives mp18 and mp19 rf DNA as specified in Table 6. Nucleotide sequencing was carried out using the dideoxy sequencing method according to the strategy outlined in Fig. 4, which is the restriction map and sequencing strategy for the 4.39 Kb BamHl fragment containing the ∝-glucosidase gene. The arrows indicate the direction and extent of nucleotide sequence determinations. Symbols denoting restriction endonuclease recognition sites are B, BamHl; H, HindIII; M, MluI; N, NdeI; P, PstI; S, SalI; X, XhoI; Pv; Pvul. The vertical lines indicate the positions of restriction sites and the o indicate use of custom made synthetic oligonucleotides as primers in extension reactions. This was carried out using either the double stranded plasmid DNA or single stranded phage DNA as specific templates and universal, reverse or custom made oligonucleotides as primers in the extension reactions.

The complete sequence of the 4.39 Kb of cloned DNA which contains the ∝-glucosidase gene is shown in Fig. 5. The first nucleotide of the BamHl site at the extreme left of the fragment (Fig. 1) was set as nucleotide 1 of the sequence. A single open reading frame (ORF 1) of 3,210 bp extending from nucleotide 170 to 3,380 was identified in

reading phase 1. A second open reading frame ORF2 was identified in reading phase 2 of the nucleotide sequence which extended from nucleotide 3,858 to 4,368. No introns were detected. Because the entire coding sequence of the peptide encoded by ORF 2 lies downstream from the MluI restriction endonuclease recognition site (Fig. 2) and because the sequences downstream from this site are not required for the expression of α-glucosidase activity in S.cerevisiae transformants, we conclude that the peptide corresponding to ORF 1 represents the C.tsukubaensis α-glucosidase gene. The function of the gene encoded by ORF 2 is not known.

Analysis of the α-glucosidase coding region

The predicted amino acid sequence of the α-glucosidase gene encoded by ORF1 is as shown in Fig. 5. Assuming that translation initiation begins at the first in-frame AUG at the extreme 5' end of the mRNA, as proposed by the Ribosome Scanning Model for translation initiation in eukaryotes: Kozak; Nucl. Acids. Res., 9 (20), (1981) 5233-5250, then translation of the α-glucosidase mRNA, must begin at nucleotide 170 and proceed through to and terminate at the first in-frame stop codon located at nucleotide 3,380 to encode a single polypeptide of 1,070 amino acids with a calculated molecular weight of 119 KDa.

Analysis of the nucleotide sequence of ORF 1 shows that there are 24 potential glycosylation sites as defined by the sequences Asn-X-Ser or Asn-X-Thr (Fig. 5). This was consistent with the results of Endoglycosidase H experiments which demonstrated that the cloned α-glucosidase gene which is expressed and secreted by S.cerevisiae is extensively glycosylated. It is not known if all of these sites are actually glycosylated.

The deduced protein coding sequence of ORF 1 was searched for regions showing homology to the amino acid sequences found at the N-termini of peptide 1 and peptide 2 (Fig. 6). Two regions of extensive sequence homology were clearly identified, namely, 9 of the 14 residues assigned to peptide 1 were homologous to amino acids 36-49 of ORF 1 while 17 of the 22 residues assigned to peptide 2 were homologous to amino acids 613-634 of ORF 1. If it is assumed that any mismatches are due to protein sequencing artifacts, then the extent of sequence homology is such that the amino acid sequences shown in Fig. 6 which have a been deduced from ORF1 must be identical with those found at the N-termini of peptides 1 and 2.

In Fig. 6, regions within α-glucosidase precursor protein (ORF 1) showing homology to the N-terminal amino acid sequences of peptide 1 and peptide 2 are shown . The numbers refer to the position of amino acids within the sequence and the * indicate matching amino acids.

Therefore, ORF 1 appears to encode a precursor polypeptide which is processed in a series of post-translational proteolytic processing steps to yield peptides 1 and 2. If proteolytic processing occurs at or around the areas showing sequence homology to the amino acid residues at the N-termini of peptide 1 and peptide 2, then ORF 1 must be processed to yield two polypeptides, the larger of which corressponds in size to peptide 1 and extends from amino acid residues 36-613 with a predicted molecular weight of 64 KDa. A second polypeptide corresponding in size to

peptide 2 extends from amino acid residues 613-1017 of ORF 1 and has a predicted molecular weight of 51.1 KDa.

Such a process step would, of course, necessitate removal of a short peptide of 36 amino acid residues from the N-terminal end of ORF 1. In view of the fact that the mature α-glucodidase is found in the extracellular fraction of S.cerevisiae cultures it is possible that this N-terminal peptide may be acting as a signal sequence. The amino acid sequence of the N-terminal extension of ORF 1 was screened for properties typical of yeast signal sequences, as exemplified by the yeast PHO 5 encodes acid phosphatase described by Meyhack et al., EMBO J, 1 (6) 675-680 (1982), and the SUC 2 encoded invertase described by Taussig and Carlson, Nucl. Acids Res. 11(6), 1943-1954 (1983).

The proposed signal sequence shared the following typical features with classical signal sequences as described by Perlman & Halvorson, J. Mol. Biol. 167(1983), 391-409, and by Von Heijne, J. Mol. Biol. 184,(1985), 99-105:

(a) a positively charged N-terminal amino acid region (containing one Lys and one Arg residue);

(b) a central hydrophobic core sequence; and

(c) a more polar carboxy terminal region which defines the cleavage site. In the case of the α-glucosidase protein, the hydrophobic core sequence ends with the sequence Leu-Ala-Leu at amino acid residues 24-26 and is following by a short stretch of

hydrophilic amino acid residues (extending from amino acid residues 26-35)

Analysis of the codon usage patterns of the α-glucosidase precursor and of the flanking transcriptional and translational regulatory sequences, confirms that the C. tsukubaensis α-glucosidase gene sequence contains many of the canonical features associated with highly expressed S. cerevisiae genes.

The Genbank and NBRF/PIR protein sequence databases containing 12,940 and 8,587 individual sequence entries, respectively, were searched for proteins showing homologies to the cloned α-glucosidase defined by ORF 1 and the unidentified protein defined by ORF 2. Homology scores were assigned according to the number of direct amino acid matches with credit given for conservative amino acid changes and downweightings where gaps were introduced to allow for sequence alignments.

No entry was identified which showed significant homology to the polypeptide encoded by ORF 2 and it is not, therefore, possible to speculate as to the possible identity or biological role of ORF2. In the case of ORF 1, however, one high score indicating significant matching of sequences was obtained from both database searches. The sequence showing homology to ORF 1 is the sucrase-isomaltase complex from rabbit intestinal mucosa; Hunziker; et al. Cell, 46, (1986) 227-234.

This sucrase-isomaltase complex (EC 3.2.1.48) is synthesized as a single chain precursor (pro SI) of approximately 260,000 daltons which possesses both enzymatic activities. This precursor is proteolytically processed by the action of pancreatic proteases into two

individual subunits, isomaltase (of approximately 140 KDa daltons corresponding to the amino terminal portion of proSI) and sucrase (of approximately 120 KDa daltons).

The promoter

Analysis of the DNA sequence encoding the C.tsukubaensis α-glucosidase gene suggests that there is an efficient yeast promoter at the extreme 5' end of the 4.39Kb BamHl fragment. This has been confirmed by experiment. We have therefore provided a means of directing the expression of other heterologous genes in S.cerevisiae, making use of the fragment.

The fact that the gene comprises a yeast promoter is shown by the ability of the BamHl-Xhol fragment, which extends from nucleotide 1-272, to direct the expression of a foreign gene (the β-galactosidase of Escherichia coli) in S.cerevisiae.

This was confirmed using an E.coli-yeast shuttle vector, YEp 365, in which the lacZ gene is inactive due to lack of an initiator methionine codon and absence of a promoter (Myers et al., Gene, 45, 299-310, 1986). This vector will express β-galactosidase in yeast only when the lacZ gene is fused in-phase to another gene possessing its own promoter. As S.cerevisiae has no endogenous β-galactosidase activity, the quantity of enzyme produced by the hybrid vector in yeast is a function of the strength of the promoter used. Thus, an efficient yeast promoter at 5' end of the 4.3Kb BamHl fragment would lead to in-frame fusion of the 272bp BamHl-Xhol fragment to the lacZ gene in YEp 365 to result in the production of large amounts of β-galactosidase.

This 272bp fragment containing 170 nucleotides of 5'non-coding sequence and 34 amino acids of the α-glucosidase gene was cloned as a BamH1 fragment into the unique BamH1 site of YEp365 and the resulting recombinant plasmid was designated pGA8904. pGA8904 contains the α-glucosidase gene fused in-frame to lacZ. S.cerevisiae AH22 cells transformed with pGA8904 formed blue colonies on chromogenic X-gal agar plates, indicating that the 272bp fragment does contain an active yeast promoter. Quantitative determinations of promoter activity were made by colorimetric assay of β-galactosidase activity in permeabilised cells which had been cultured for 3 days at 24°C in selective minimal medium. The α-glucosidase fragment directed the synthesis of appreciable levels of β-galactosidase activity in S.cerevisiae AH22 transformants and the enzyme levels were comparable to those produced by the action of the S.cerevisiae alcohol dehydrogenase (ADC1) promoter (Table 1). The ADC1 promoter fragment was derived from the yeast expression vector pAH9 (Ammerer, G., Methods in Enzymol., 101, (1983) 192-201) and contains 1.5Kb of 5' non-coding sequence in addition to the first three amino acids of the alcohol dehydrogenase gene on a Bam HI - Hind III fragment. This fragment was inserted into the Bam HI - Hind III cloning sites of YEp 365 to give pTAG 3. No β-galactosidase activity was detected in yeast cells transformed with the vector plasmid alone.

The results obtained are shown in Table 1 below:

TABLE 1

Assessment of C.tsukubaensis α-glucosidase promoter strength

Recombinant plasmid in S.cer. AH22	Promoter fragment	Vector	ß-galactosidase activity (units/O.D.$_{600nm}$ ml^{-1} /min)
pGA8904	α-glu (272bp BamH1-Xho1)	YEp 365	77.4
pTAG3	ADC1 (1.5Kb BamH1-Hind111)	YEp 365	73.5

TABLE 2

SUBSTRATE SPECIFICITIES OF CLONED ENZYME ENCODED BY

S.cerevisiae AH22 (pGA8701)

Substrate (2% w/v in McILvaine buffer, pH 5.4)	Relative Activity (%)	Sugar Linkage Type	Sugar Unit Number
Maltose	100	α(1-4)	2
Maltotriose	59	α(1-4)	3
Maltotetraose	48	α(1-4)	4
Sucrose	55	Fructosyl-α-D-glucoside α-(1-2)	2
Panose	55	α(1-4), α(1-6)	3
Isomaltose	35	α(1-6)	2
Phenyl α-D-glucoside	18	Aryl-A*-D-glucoside	1
Melezitose	8.8	α(1-2), α(1-3)	3
Amylose	7.5	α1-4)	Polymer
Dextrin (Difco)	8.0	α(1-4), α(1-6)	Polymer
Dextrin (B.D.H.)	6.8	α(1-4), α(1-6)	Polymer
Dextrin (Puriss)	6.4	α(1-4), α(1-6)	Polymer
Turanose	4.1	α(1-3)	2
Glycogen	0.95	α(1-4), α(1-6)	Polymer
α-methyl-D-Glucoside	0.7	Alkyl-α-D-glucoside	1
Starch (Lintners)	0.5	α(1-4), α(1-6)	Polymer
Starch (Potato)	0	α(1-4), α(1-6)	Polymer
Starch (corn)	0	α(1-4), α(1-6)	Polymer
Amylopectin	0	α(1-6)	Polymer
Pullulan	0	α(1-6)	Polymer
Trehalose	0	Glucopuranoside	2
α-cyclodextrin	0	Cyclic dextrin	Polymer

TABLE 3

EFFECT OF pH ON α-GLUCOSIDASE ACTIVITY

	pH						
	3.4	3.9	4.2	4.4	4.6	4.9	5.4
Enzyme Activity (units/ml)	5.53	6.96	7.17	7.26	6.97	6.67	5.03
% Relative Activity	75.95	96.11	99.40	100.00	96.10	92.0	69.38

TABLE 4

EFFECT OF TEMPERATURE ON α-GLUCOSIDASE ACTIVITY

	INCUBATION TEMPERATURE (°C)							
	20	40	45	50	55	58	60	65
Enzyme activity (units/ml)	1.1	3.1	3.7	4.5	5.5	5.7	5.6	2.3
Relative activity (%)	19.3	53.5	64.0	79.0	96.5	100.0	99.1	40.4

TABLE 5.

THEMOSTABILITY OF THE CLONED α-GLUCOSIDASE AT 60°C

	PREINCUBATION TIME (MIN) AT 60°C						
	0	1	2	5	10	20	60
Enzyme Activity (units/ml)	5.39	3.58	0.98	0	0	0	0
Relative Activity (%)	100	66.42	17.63	0	0	0	0

Table 6

Parent plasmid	C.tsukubaensis restriction fragment	Restriction enzymes used to excise fragments for cloning into M13 mp18/mp19 rf DNA	M13 plasmid derivative
pTS001	Sal1-Sal1	Sal1	pSS1901
pTS003	BamH1-Sal1	Hind111+BamH1	pHB1803 pHB1903
pTS004	BamH1-Xho1	Hind111+BamH1	pHB1804 pHB1904
pTS005	Sal1-Sal1	Sal1	pSS1905
pTS007	Sal1-Hind111	Hind111+BamH1	pHB1807 pHB1907
pTS009	Hind111-BamH1	Hind111+BamH1	pHB1809 pHB1909
pTS010	Sal1-Hind111	Hind111+BamH1	pHB1810 pHB1910
pTS011	Pst1-Sal1	EcoR1+Pst1	pEP1811 pEP1911
pTS013	Xho1-Pst1	EcoR1+Pst1	pEP1813 pEP1913
pTS014	Hind111-Nde1	Hind111+BamH1	pHB1814 pHB1914
pTS015	Nde1-BamH1	BamH1	pBB1815

The recombinant bacteriophage M13 based mp18 and mp19 plasmid derivatives (column 4) were constructed by excising the restriction fragments (column 2) from the parent pUC8 recombinant plasmids (column 1) with the specific restriction endonucleases (column 3) and then cloned into homologous sites within the mp18 and mp19 rf DNA.

CLAIMS

1. Saccharomyces cerevisiae having an α-glucosidase gene from Candida tsukubaensis CBS 6389.

2. A method of providing Saccharomyces cerevisiae with enhanced α-glucosidolytic activity comprising transforming said Saccharomyces cerevisiae with an α-glucosidase gene from a genomic library prepared from Candida tsukubaensis.

3. A method of fermenting carbohydrate material comprising adding a culture of S.cerevisiae to an aqueous solution, suspension or dispersion of said carbohydrate material wherein S.cerevisiae has an α-glucosidase gene derived from Candida tsukubaensis CBS 6389 .

4. A 4.39 Kb Bam Hl fragment having the nucleotide sequence set out in Fig. 5 of the accompanying Drawings.

5. A Bam Hl-Xhol fragment extending from nucleotide 1-272 of the above-identified 4.39 Kb Bam Hl fragment.

6. α-glucosidase obtained in an extracellular fraction of a culture of S.cerevisiae according to Claim 1.

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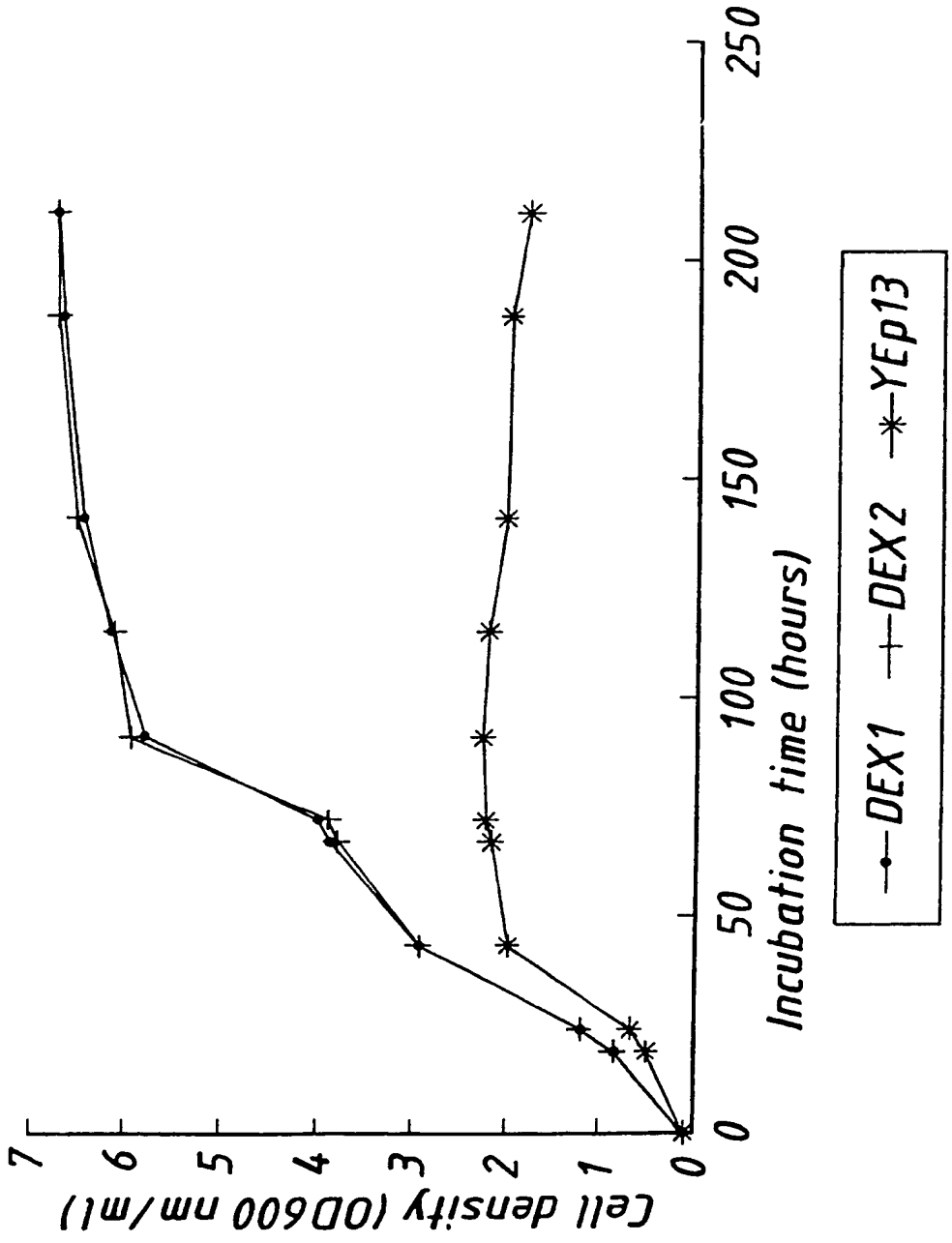
FIG.1(a)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1
2
3
4
5
6
7
0
50
100
150
200
250
Incubation time (hours)
DEX1
DEX2
YEp13

FIG.1(b)

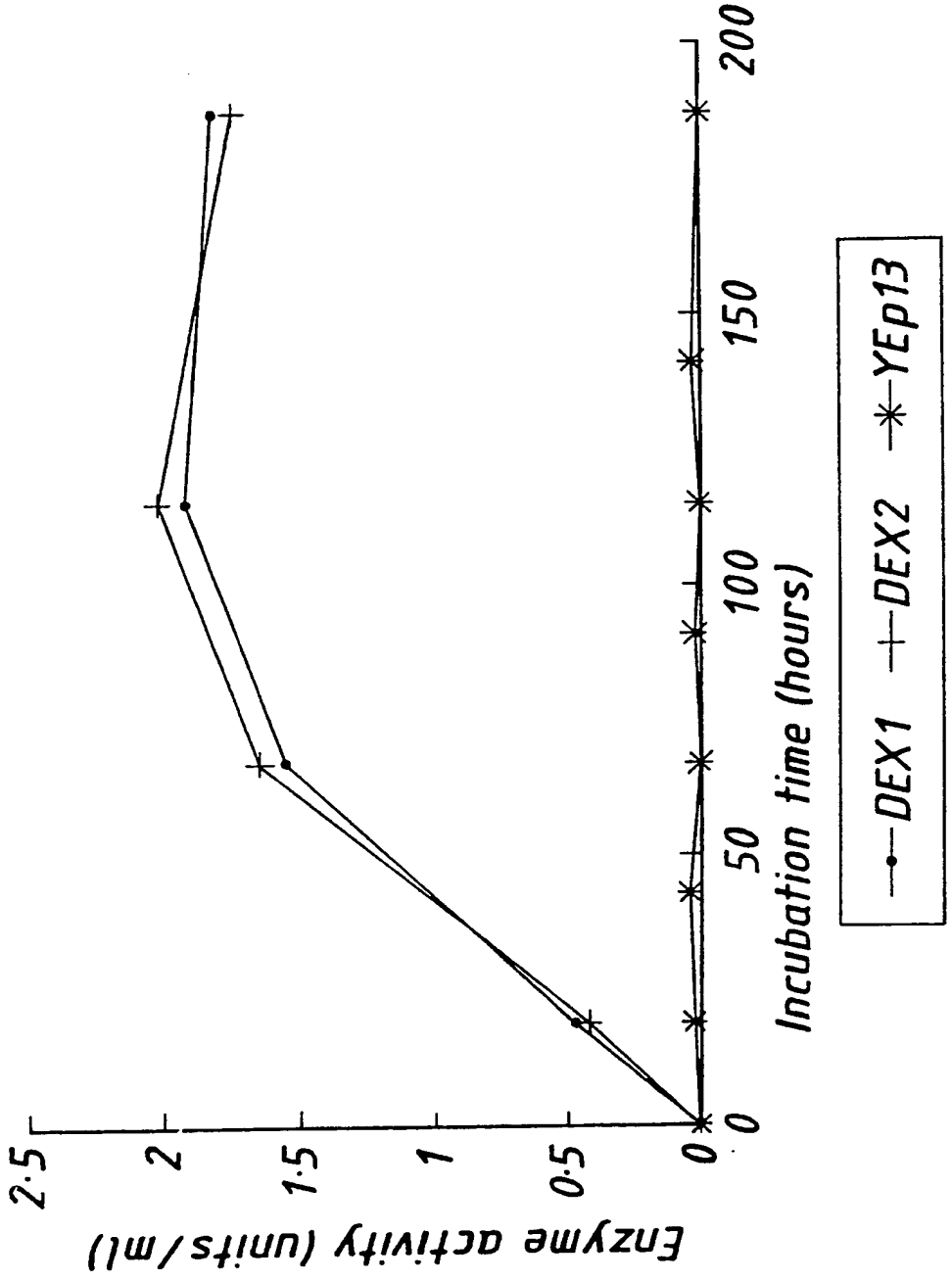

Enzyme activity (units/ml)
2·5
2
1·5
1
0·5
0
0
50
100
150
200
Incubation time (hours)
DEX1
DEX2
YEp13

FIG. 2.

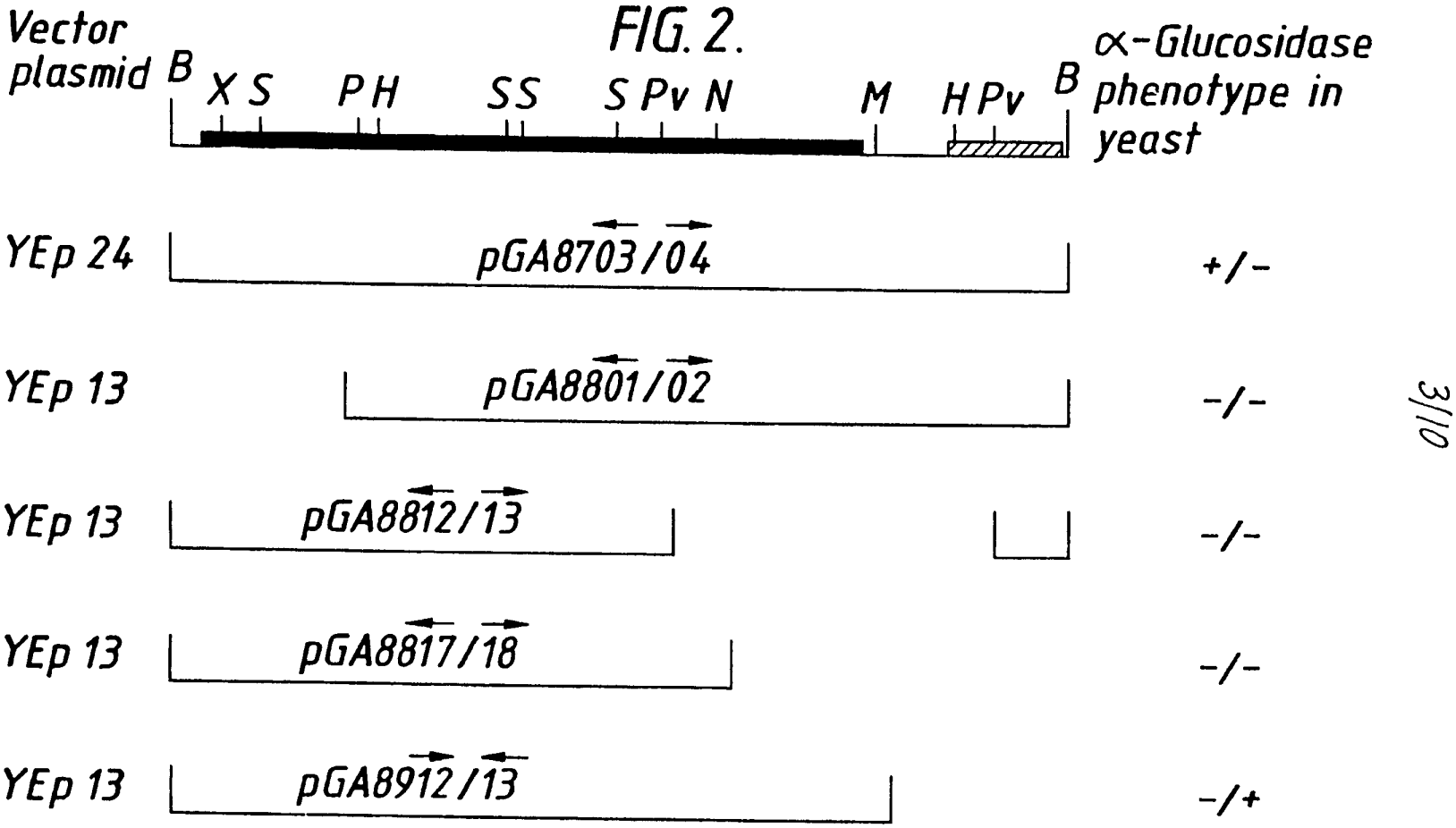
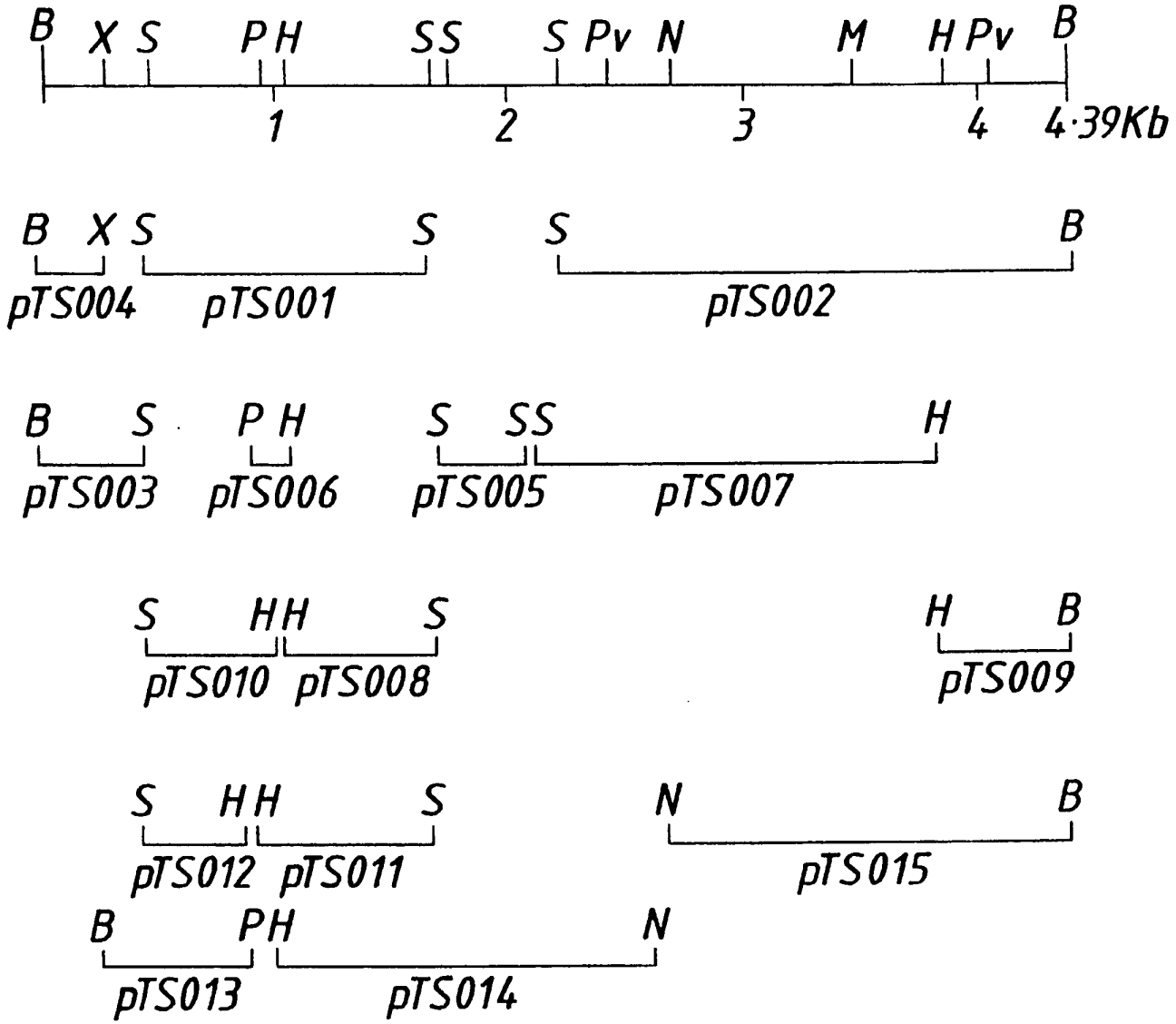

Vector plasmid
B X S P H S S S Pv N M H Pv B
∝-Glucosidase phenotype in yeast
YEp 24
pGA8703/04
+/-
YEp 13
pGA8801/02
-/-
YEp 13
pGA8812/13
-/-
YEp 13
pGA8817/18
-/-
YEp 13
pGA8912/13
-/+

FIG. 3.


B X S P H S S S Pv N M H Pv B
1
2
3
4
4·39Kb
B X S S
pTS004
pTS001
S B
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B S
pTS003
P H
pTS006
S S S H
pTS005
pTS007
S H H S
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pTS014

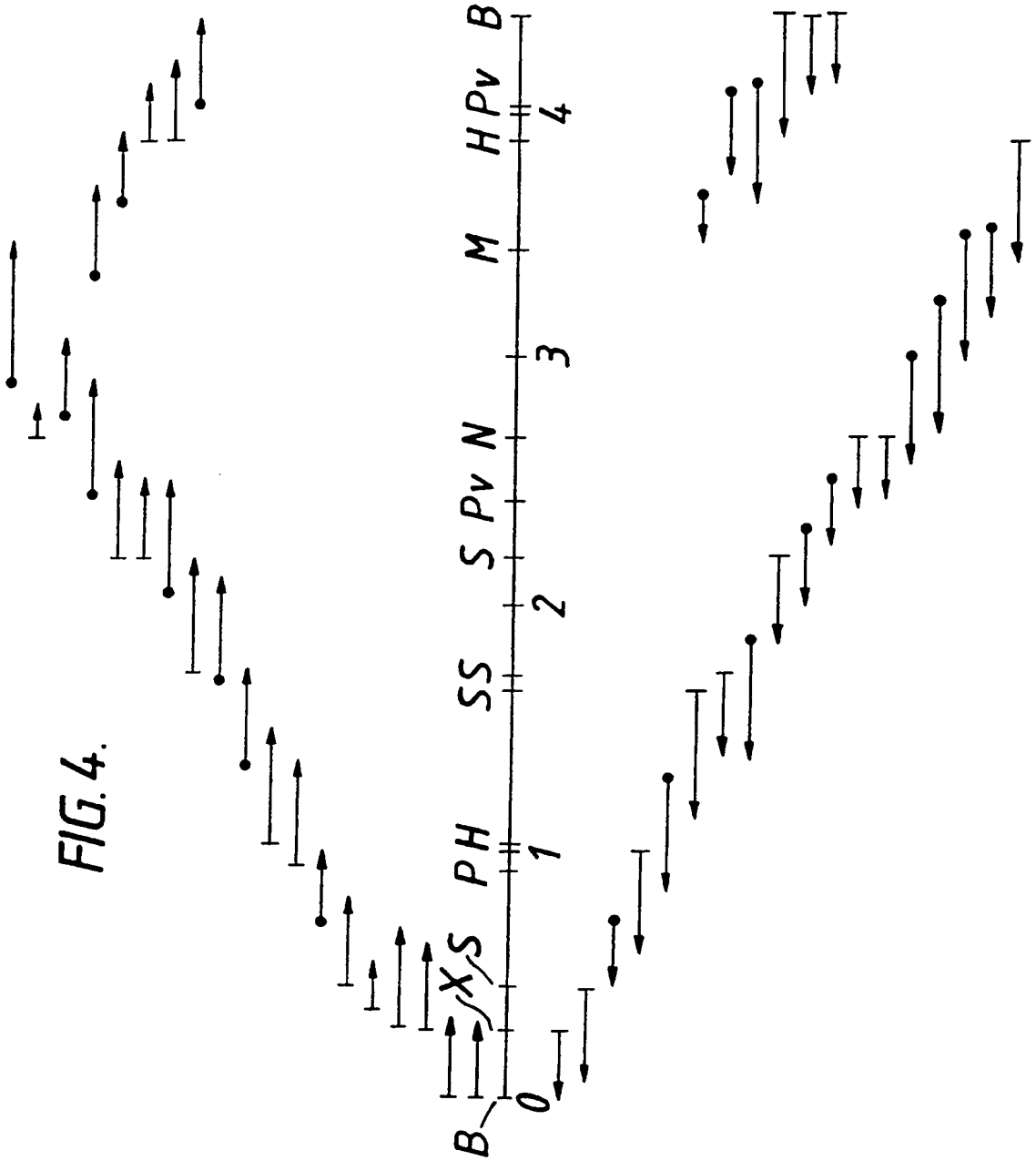

FIG. 4.

FIG. 5 NUCLEOTIDE SEQUENCE OF 4.39 Kb BamH1 FRAGMENT

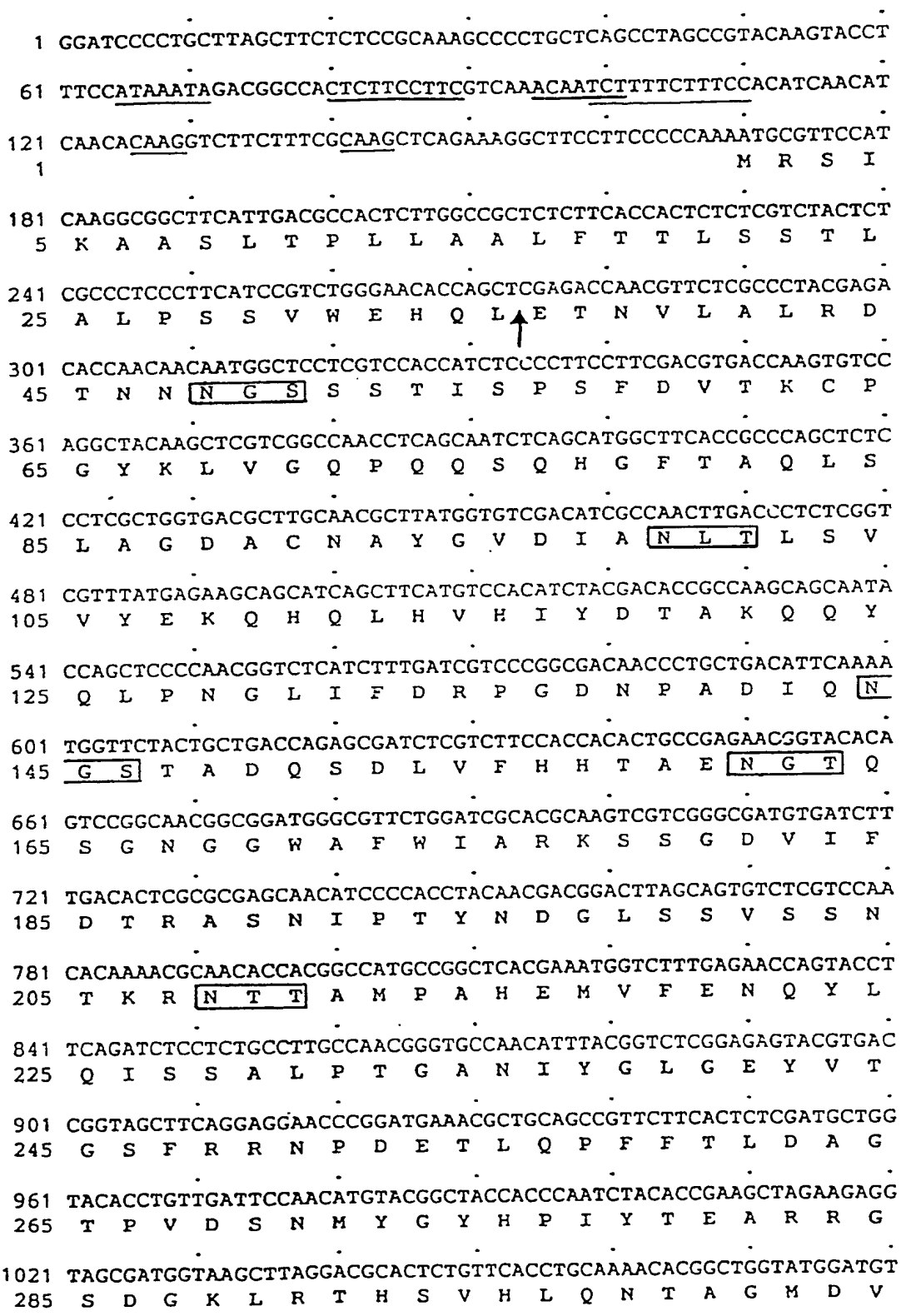

FIG.5 Cont.


```
1081 GCTTCTGCGTAGGGGTGTCATTCAGTACAGGGCGATTGGCGGTACGCTCGACTTCCGATT
 305  L  L  R  R  G  V  I  Q  Y  R  A  I  G  G  T  L  D  F  R  F

1141 CTTTTCTGGTGACCAGCCTGCTTCCTCTTCTTCGTCTTCGAGCGGAAACGATAAGGCAGT
 325  F  S  G  D  Q  P  A  S  S  S  S  S  S  S  G  N  D  K  A  V

1201 TGCAACCGTAAAGAACAGCCCGAACACTGCCATTCAGCAGTACGTCAACTTCATTGGCAA
 345  A  T  V  K  N  S  P  N  T  A  I  Q  Q  Y  V  N  F  I  G  N

1261 CCCCGTCATTCACCCTTACTGGTCGTATGGCTTCCACCTCTGCCGATGGGGCTACAACAA
 365  P  V  I  H  P  Y  W  S  Y  G  F  H  L  C  R  W  G  Y  N [N]

1321 CGTTTCCGAGACCCAAGCCGTCATCGACGCCATGCGACAGAACAACATCCCCCTCGAAGT
 385 [V  S] E  T  Q  A  V  I  D  A  M  R  Q  N  N  I  P  L  E  V

1381 GCAATGGAACGATATCGACTACTTGCAAGAGTTCCGTGACTTTACCACGGACCCGCAACG
 405  Q  W  N  D  I  D  Y  L  Q  E  F  R  D  F  T  T  D  P  Q  R

1441 ATTCCCTCAGAAAGAGTTTGCTGCTATGATTGCCAAGCTCAAGGATAACCACCAGCACTA
 425  F  P  Q  K  E  F  A  A  M  I  A  K  L  K  D  N  H  Q  H  Y

1501 CATTCCTATCATCGACATGGCTATTCCTAAGGCTCCGACGAACGACACCGACGTCTACTA
 445  I  P  I  I  D  M  A  I  P  K  A  P  T [N  D  T] D  V  Y  Y

1561 TCCTGGTACTCGTGGCGATGAGCTCGACGTCTTCATCAAGAATCGCAACGGCTCGCAATA
 465  P  G  T  R  G  D  E  L  D  V  F  I  K  N  R [N  G  S] Q  Y

1621 CATCGGTGAAGTTTGGCCCGGTTACACCAACTTTGTCGACCAACAGGCCGAGAATGCGGG
 485  I  G  E  V  W  P  G  Y  T  N  F  V  D  Q  Q  A  E  N  A  G

1681 CAAGTGGTGGACCGAGGCGATCCGCAACTTTAGCGAGATTGTCGACTTCTCAGGTATCTG
 505  K  W  W  T  E  A  I  R [N  F  S] E  I  V  D  F  S  G  I  W

1741 GCTGGATATGAACGAGCCTTCGAGTTTCGTTATCGGTAATGCTGCTGGGCCGGAGACGAA
 525  L  D  M  N  E  P  S  S  F  V  I  G  N  A  A  G  P  E  T [N]

1801 TTTGTCGAACACGCCAGCCTATACTGCGGCGACGAGCGTTGCTGGATGGCCGCAGGGTTA
 545 [L  S] N  T  P  A  Y  T  A  A  T  S  V  A  G  W  P  Q  G  Y

1861 CAACAACCTCACTTGGGGCACTTCGGGTAATATCACCGTCAACGGCAGTTACACTTACCA
 565  N [N  L  T] W  G  T  S  G [N  I  T] V [N  G  S] Y  T  Y  Q

1921 GCAAGGACCTGTTCAGAACAACGACGGCTCGAAGCAGCGTCGTTCGCTTCTGCTTTCGCG
 585  Q  G  P  V  Q  N  N  D  G  S  K  Q  R  R  S  L  L  L  S  R

1981 CGACGAAGATGTTCTCGTCCAGCGTGACATCAACGTCAACGGTGGCAATGGCGACAAGTT
 605  D  E  D  V  L  V  Q  R  D  I  N  V  N  G  G  N  G  D  K  F

2041 TGGCCCCGAAGATCCCAACTATCAATACGCTAACTCTTCGCAGCGATACCTCTCCAACCC
 625  G  P  E  D  P  N  Y  Q  Y  A [N  S  S] Q  R  Y  L  S  N  P

2101 TCCCTACGCCATCCACAACGGTATCCACATCAGCGAAACCCCCCCTTAACGTCAACTTGGA
 645  P  Y  A  I  H  N  G  I  H  I  S  E  T  P  L  N  V  N  L  D
```

FIG.5 Cont.


```
2161 TAAGAAGACCGTTGCTATGGAAGCTGTTGGTGTCGACGGTCAACGCGCTTTCTACGACGT
 665  K  K  T  V  A  M  E  A  V  G  V  D  G  Q  R  A  F  Y  D  V

2221 TCACAATCTTGATGGCACCCTGGAGGAGCAACACTTCTACAACGCTCTTCGCGATATTCG
 685  H  N  L  D  G  T  L  E  E  Q  H  F  Y  N  A  L  R  D  I  R

2281 CCCGCAAGAACGACCGTTCCTCATCTCACGTTCTACCTACCCTGGTGCAGGAAAGTTCAC
 705  P  Q  E  R  P  F  L  I  S  R  S  T  Y  P  G  A  G  K  F  T

2341 CGGTCATTGGTTGGGTGACAACTATGCGCTTTGGACCATCTTGCCCGGCGAAGAGGCGTA
 725  G  H  W  L  G  D  N  Y  A  L  W  T  I  L  P  G  E  E  A  Y

2401 CAAAGCTGGCGCTGGTATGGCGCAGTCGATCGACGGTGTACTCCAGTTCCAGATCTTTGG
 745  K  A  G  A  G  M  A  Q  S  I  D  G  V  L  Q  F  Q  I  F  G

2461 CATCCACCTTATTGGAGCCGACATTTGCGGATTTAACCGTAACAGTGACGAAGAGCTGTG
 765  I  H  L  I  G  A  D  I  C  G  F  N  R  N  S  D  E  E  L  C

2521 CAATCGTTGGATGATGCTTGGTGCATTCTTGCCGTTCATGCGCAACCACAATACGATTGG
 785  N  R  W  M  M  L  G  A  F  L  P  F  M  R  N  H  N  T  I  G

2581 TGCGATTGCTCAGGAGCCTTTCCGATGGGACAGTGTTGCCAACGCGAGCAGGATCGCGAT
 805  A  I  A  Q  E  P  F  R  W  D  S  V  A [N  A  S] R  I  A  I

2641 CAACAAGAGGTACGAGATCTTGCCTTCGCTGTATTCGCATATGGCTCAGAGCGCCGAATC
 825  N  K  R  Y  E  I  L  P  S  L  Y  S  H  M  A  Q  S  A  E  S

2701 GGGCGAACCAGCGGTGAGGGCGCTGTGGTACGAGTTCGATGAGGTTTTCGAACAGACCAA
 845  G  E  P  A  V  R  A  L  W  Y  E  F  D  E  V  F  E  Q  T  K

2761 GGATTACGCTCATCAGTTCTTGTTCGGAGACGACTTGTTGGTCAGTCCAGTGTTGGAGCC
 865  D  Y  A  H  Q  F  L  F  G  D  D  L  L  V  S  P  V  L  E  P

2821 CAATGTGACGCAGATCAAGGCGTTGTTCCCTAACGCCGGCGGAAAGTGGAGGAATGTTTT
 885 [N  V  T] Q  I  K  A  L  F  P  N  A  G  G  K  W  R  N  V  F

2881 CAGCTACGAAGCGCTCGACGTTGAATACAATAAGAACGTAACCGTTGATGCGGCGCTTTC
 905  S  Y  E  A  L  D  V  E  Y  N  K [N  V  T] V  D  A  A  L  S

2941 GACGATCAACGTCCACCTTCGACCAGGAAAGGTTCTCCTCACCCACTCCAAACCCGCGTA
 925  T  I  N  V  H  L  R  P  G  K  V  L  L  T  H  S  K  P  A  Y

3001 CACGGTCTACGAAACCGCTCAAAGCCCCTACGGCCTCATTGTCAACTTGAACGACCAAGG
 945  T  V  Y  E  T  A  Q  S  P  Y  G  L  I  V  N  L  N  D  Q  G

3061 AGAGGCAAAGCAAACCTTCTACCTCGATGACGGTATGACACCCGCCCCCACCCCTAACTC
 965  E  A  K  Q  T  F  Y  L  D  D  G  M  T  P  A  P  T  P [N  S]

3121 GACACTTACCGTAAGCGCAGGTAACAACTCGGTCAACGGAAGCATCGAAGGAGAGTACAA
 985 [T] L  T  V  S  A  G [N  N  S] V [N  G  S] I  E  G  E  Y  K

3181 GGCGCAGCAAAACTTGACCTACGTTGTCGTGCTCGATGTCAAGCAGAAGCCAACCCAGGT
1005  A  Q  Q [N  L  T] Y  V  V  V  L  D  V  K  Q  K  P  T  Q  V
```

individual subunits, isomaltase (of approximately 140 KDa daltons corresponding to the amino terminal portion of proSI) and sucrase (of approximately 120 KDa daltons).

The promoter

Analysis of the DNA sequence encoding the C.tsukubaensis α-glucosidase gene suggests that there is an efficient yeast promoter at the extreme 5' end of the 4.39Kb BamH1 fragment. This has been confirmed by experiment. We have therefore provided a means of directing the expression of other heterologous genes in S.cerevisiae, making use of the fragment.

The fact that the gene comprises a yeast promoter is shown by the ability of the BamH1-Xho1 fragment, which extends from nucleotide 1-272, to direct the expression of a foreign gene (the β-galactosidase of Escherichia coli) in S.cerevisiae.

This was confirmed using an E.coli-yeast shuttle vector, YEp 365, in which the lacZ gene is inactive due to lack of an initiator methionine codon and absence of a promoter (Myers et al., Gene, 45, 299-310, 1986). This vector will express β-galactosidase in yeast only when the lacZ gene is fused in-phase to another gene possessing its own promoter. As S.cerevisiae has no endogenous β-galactosidase activity, the quantity of enzyme produced by the hybrid vector in yeast is a function of the strength of the promoter used. Thus, an efficient yeast promoter at 5' end of the 4.3Kb BamH1 fragment would lead to in-frame fusion of the 272bp BamH1-Xho1 fragment to the lacZ gene in YEp 365 to result in the production of large amounts of β-galactosidase.

FIG.5 Cont.

```
4321 GTATCACAGATCTCATGGATGTTGTAAGTCTTTCTCTGCCACCATCTTGAAATCCCTTTC
 156   I  T  D  L  M  D  V  V  S  L  S  L  P  P  S  X

4381 TGGAGAGCGGATCC
```

FIG.6

```
Peptide 1     1ATNVLALFETQNPG14
                ******  * * *
ORF 1        36ETNVLALRDTNNNG49

Peptide 2     1NIYVNGGNVDKFGPELHNYQYA22
                * ***** ******  *****
ORF 1       613DINVNGGNGDKFGPEDPNYQYA634
```